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Uniport of monoanionic L-malate in membrane vesicles from *Leuconostoc oenos*

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L-malate transport was studied in membrane vesicles from *Leuconostoc oenos* MLE(−) (mutant lacking malolactic enzyme) which were fused with liposomes containing beef heart cytochrome *c* oxidase as a proton-motive-force-generating system. In these hybrid membranes, accumulation of L-malate was observed in response to a pH gradient (ΔpH), with the inside alkaline, but was strongly inhibited by a membrane potential ($\Delta\Psi$) of normal polarity (inside negative). Imposition of a $\Delta\Psi$, with the inside positive, by means of valinomycin-mediated potassium influx, resulted in a rapid accumulation of L-malate, indicating that L-malate was taken up in an anionic form. The results are consistent with a uniport mechanism facilitating the uptake of monoanionic L-malate, the dominant species at the low pH of the experiments. Kinetic analysis of ΔpH-driven L-malate uptake in the pH range 3.0–5.8, yielded apparent affinity constants that varied less than twofold when calculated on the basis of the concentrations of monoanionic L-malate, whereas the values differed 2–3 orders of magnitude for the other species. At L-malate concentrations above 1 mM, a non-saturable transport component became apparent which may reflect passive influx of L-malic acid. Substrate specificity studies indicated that citrate and L-malate (and possibly D-lactate and L-lactate) compete for a single general carboxylate transport system. The carboxylate transport system catalysed homologous L-malate and heterologous L-malate/citrate exchange with rates similar to the rate of L-malate efflux. Since metabolic energy is conserved during malolactic fermentation in *L. oenos*, the underlying mechanism most likely involves electrogenic monoanionic L-malate uptake, in combination with H^+ consumption in the cytoplasm, followed by diffusion outwards of lactic acid plus carbon dioxide.

Malolactic fermentation is performed by some lactic acid bacteria and, in the process, metabolic energy is conserved. The pathway involves the uptake of L-malate, which is stoichiometrically converted to L-lactic acid plus CO_2, after which the reaction products leave the cell. The $\Delta G^{\circ\prime}$ of the L-malate decarboxylation reaction at pH 7.0 and pCO_2 10^5 Pa is approximately −26.5 kJ/mol [6]. Part of the free energy (ΔG), taking the $\Delta G^{\circ\prime}$ of the reaction and the concentrations of the reactants into account, may be conserved in a chemiosmotic coupling mechanism [14]. Such a mechanism leads to the generation of a proton motive force (Δp) if the overall transport reaction is electronegative and decarboxylation (proton consumption) takes place in the cytoplasm. This type of conservation of metabolic energy has already been described for the decarboxylation of oxalate in *Oxalobacter formigenes* [1], L-malate in *Lactococcus lactis* [15] and *Lactobacillus plantarum* [11] and histidine in *Lactobacillus buchneri* [10]. In the case of decarboxylation of oxalate and histidine, the corresponding transport reactions have been shown to proceed as oxalate/formate exchange and histidine/histamine exchange or, in more general terms, as electrogenic precursor/product exchange [13]. For malolactic fermentation in *L. lactis*, electrogenic malate/lactate exchange was demonstrated but the same transport system was also shown to catalyse electrogenic monoanionic L-malate (L-malateH^-) uniport, at least *in vitro* [15]. In the case of *L. plantarum*, some evidence has been presented for transport of L-malateH^- with and without protons [11]. These latter studies, however, are complicated by the rapid metabolism of L-malate, and may therefore not have revealed a L-malate/L-lactate exchange reaction.

Leuconostoc oenos is well known for its ability to carry out malolactic fermentation and for this reason the organism is frequently used in the deacidification of wine. The malolactic enzyme of *L. oenos* has recently been isolated from the cytoplasmic fraction of the cells and purified to homogeneity (I. Capucho, personal communication). In this study, we have examined the mechanism of L-malate uptake by *L. oenos*. Most of the experiments were carried out at relatively low pH values, i.e. comparable to the conditions in wine fermentation. It is shown that L-malate uptake proceeds efficiently as electrogenic monoanionic L-malate uniport, thereby generating a membrane potential (inside negative). The driving

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Abbreviations. Malolactic enzyme, L-malate:NAD^+ oxidoreductase (oxaloacetate decarboxylating enzyme); L-malateH_2, L-malic acid; L-malateH^-, monoanionic L-malate (or monocarboxylate anion of L-malate); L-malate^{2-}, dianionic L-malate (or dicarboxylate anion of L-malate).

Enzyme. Malolactic enzyme (EC 1.1.1.38).

force for L-malate uptake is supplied by the L-malateH^- concentration gradient which results from the rapid metabolism of L-malate inside the cell.

MATERIALS AND METHODS

Strain and growth conditions

L. oenos Lo 84.13 MLE(−) (mutant lacking malolactic enzyme) was kindly provided by J. F. Cavin, Laboratoire de Microbiologie, Université de Bourgogne, France. Bacterial cultures were grown in FT 80 medium, pH 4.8, [2], containing 5 g/l DL-malic acid and lacking Tween 80. Glucose (2 g/l) and fructose (8 g/l) were autoclaved separately and added to the medium just before inoculation. D,L-malic acid was added to the medium in order to express the malate transport system maximally. Cells were grown in batch cultures at 30°C and harvested at the end log phase of growth.

Preparation of membrane vesicles

Right-side-out membrane vesicles from *L. oenos* MLE(−) were prepared according to the protocol of Otto et al. (1982) [12] with some modifications; to enhance cell lysis, 8.5 U/ml mutanolysine (Sigma) was added and the amount of lysozyme was increased to 10 mg/ml. This suspension was incubated for 45 min at 30°C. A low-speed centrifugation step (750×*g*, for 1 h at 4°C) was used to remove intact cells and protoplasts from the lysis mixture. Subsequently, membrane vesicles were collected by a high speed centrifugation (48200×*g* for 30 min at 4°C) and resuspended in 50 mM potassium phosphate, pH 6.0, plus 10 mM $MgSO_4$. The isolated membrane vesicles were rapidly frozen and stored in liquid nitrogen.

Fusion of membrane vesicles with liposomes

Liposomes containing cytochrome *c* oxidase were prepared as described previously [3]. *L. oenos* membrane vesicles and the proteoliposomes (0.11 nmol cytochrome *c* oxidase/mg lipid) were mixed at a protein lipid ratio of 1:10 (by mass) and fused by freezing in liquid nitrogen, followed by slow thawing at room temperature. To form unilamellar vesicles, the thawed suspension was passed (extruded) 11-times through 200-nm polycarbonate filters (Avestin) [9]. Hybrid membranes were collected by centrifugation (250000×*g*, at 4°C for 30 min) and resuspended in 50 mM potassium phosphate, pH 5.0. The same procedure was used for the fusion of membrane vesicles with liposomes lacking cytochrome *c* oxidase.

Transport assays

Hybrid membranes were added to oxygen-saturated 50 mM potassium phosphate, 100 mM KCl, pH 5.0, supplemented with the electron donor system which was composed of 200 µM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 20 µM cytochrome *c* and 10 mM potassium ascorbate, pH 5.0. After a 1-min incubation, the transport reaction was started by the addition of L-[^{14}C]malate or L-[^{14}C]leucine. The reaction was stopped by the addition 2 ml ice-cold 100 mM LiCl and the mixture was immediately filtered through 0.45-µm pore-size cellulose nitrate membranes (Schleicher & Schüll GmbH). Filters were washed with 2 ml LiCl, placed into 2 ml scintillation liquid and the radioactivity was determined.

L-malate uptake, driven by artificial ion gradients, as well as efflux and exchange reactions were analysed in hybrid membranes devoid of cytochrome *c* oxidase. For efflux and exchange experiments, hybrid membranes were equilibrated with the appropriate concentration of labelled substrate for 2 h at 25°C. To initiate the efflux and exchange reactions, the loaded membranes were diluted 100-fold in buffer without and with counter substrate, respectively. For the generation of an artificial membrane potential ($\Delta\psi$), inside positive, the hybrid membranes were washed twice with 50 mM sodium phosphate, pH 4.0, 1 mM benzoic acid and in the presence of valinomycin (2 nmol/mg protein) to deplete the membrane vesicles of potassium. Concentrated membranes were diluted into either potassium phosphate ($\Delta\psi$, inside positive) or sodium phosphate (no gradient) containing 1 mM benzoic acid plus L-[^{14}C]malate (concentrations used are indicated in the legends to the figures), and further handling of the samples was as described above. Transport experiments at different external pH values were performed by imposing artificial H^+ gradients as described by Maloney and Hansen, (1982) [8]. Briefly, hybrid membranes were suspended in 50 mM potassium phosphate, pH 6.5, and the reaction was started by the simultaneous addition of L-[^{14}C]malate and a small volume of 0.5 M sulphuric acid to rapidly acidify the external media to the desired final pH. Samples were taken within the first 2 s. A specific internal volume of 8 µl/mg protein was assumed for the hybrid membranes [3].

Miscellaneous

The protein content was determined by the method of Lowry [6] in the presence of 0.5% (mass/vol.) sodium dodecyl sulfate using bovine serum albumin as a standard.

Chemicals

L-[U-^{14}C]malate (48 Ci/mol) and L-[U-^{14}C]leucine (312 Ci/mol) were purchased from Amersham. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Characterisation of membrane vesicles and hybrid membranes

Uptake of L-malate into whole cells is followed by a rapid conversion to lactic acid plus CO_2 and detailed information regarding the mechanism of transport of precursor and reaction products can, therefore, not be obtained. In a previous study [15], it was observed that it is difficult to isolate membrane vesicles that are totally devoid of malolactic enzyme activity. Although most of the enzyme is in the cytoplasmic fraction, a small amount of malolactic enzyme always ends up in the membrane preparation (<1%). Therefore, membrane vesicles were prepared from a strain that lacks a functional malolactic enzyme, *L. oenos* MLE(−) [2]. The membrane vesicles obtained were very leaky to ions and could not maintain artificially imposed ion gradients for more than a few seconds (data not shown). The tightness of the membrane was improved by fusing the membrane vesicles with liposomes prepared from *E. coli* phospholipids. Membrane vesicles were fused with liposomes containing cytochrome *c* oxidase in order to be able to generate a proton motive force (Δp) for prolonged periods of time.

Uptake of leucine was studied at pH 5.0 upon energization of the hybrid membranes with the electron donor system ascorbate-*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine-cytochrome *c*. Highest leucine accumulation levels were observed in the presence of a Δp composed of $\Delta\psi$ (inside negative) and ΔpH (inside alkaline) (Fig. 1A). The levels of leucine accumulation were relatively low due to the low activity of cytochrome *c* oxidase at the pH of the experiment (pH 4). The role of each of the components of Δp was investigated in more detail by performing the experiments in the presence of ionophores. Accumulation of leucine decreased upon dissipation of the membrane potential with the potassium ionophore valinomycin, and also upon dissipation of the transmembrane pH gradient with nigericin. Addition of both ionophores completely abolished leucine accumulation (data not shown). In a control experiment, in which no electron donor was present, only equilibration of leucine was observed (Fig. 1A). These experiments indicate that leucine is transported by a solute-H^+ symport system and that the hybrid membranes are suitable for analysing secondary transport in *L. oenos*.

Driving force of L-malate uptake

The driving force of L-malate uptake was analysed in the same hybrid membranes. In contrast to leucine transport, L-malate accumulation was not observed in the presence of a Δp that was composed of a $\Delta\psi$ (inside negative) and a ΔpH (inside alkaline) (Fig. 1B). However, accumulation of L-malate was observed when the $\Delta\psi$ was dissipated by valinomycin, while dissipation of the ΔpH by the K^+/H^+ ionophore nigericin resulted in complete inhibition of L-malate uptake. In the presence of nigericin, i.e. when Δp is composed of $\Delta\psi$ (inside negative) only, L-malate did not even equilibrate across the membrane, whereas it did in the absence of Δp (Fig. 1B). Although dissipation of the $\Delta\psi$ may have resulted in an increased ΔpH, it is unlikely that such an effect is the only cause for the increase in uptake (and accumulation) of L-malate. In fact, the results suggest that L-malate uptake is driven by ΔpH whereas $\Delta\psi$ may act as a counterforce. The inhibition by the membrane potential is indicative for electrogenic uptake of anionic L-malate.

Electrogenic nature of L-malate transport

If a $\Delta\psi$ (inside negative) acts as a counterforce for L-malate uptake, it should be possible to drive L-malate uptake (accumulation) by a $\Delta\psi$ of opposite polarity. A $\Delta\psi$ (inside positive) was generated by diluting K^+-free hybrid membranes (equilibrated with Na^+) in 100 mM potassium phosphate in the presence of 1 µM valinomycin. To prevent the generation of a ΔpH (inside alkaline) from the proton efflux in response to the generated membrane potential, the experiments were performed in the presence of a weak acid. L-malate accumulated under these conditions, i.e. in the presence of a $\Delta\psi$ (inside positive), while in the absence of a $\Delta\psi$ only equilibration of L-malate was observed (Fig. 2).

To substantiate the electrogenicity of L-malate uptake further, artificial H^+ gradients were imposed by rapid acidification of the medium with sulphuric acid, thereby decreasing the pH. Upon lowering the external pH of the membrane from 6.5 to 3.5, a transient pH gradient was formed together with a membrane potential (inside positive). The membrane potential was formed because protons move inwards much faster than sulfate and phosphate anions [8]. The combination of both a ΔpH (inside alkaline) and a $\Delta\psi$ (inside positive) should yield the highest driving force for L-malate uptake. Indeed, the highest levels of malate accumulation were observed under these conditions (Fig. 3). When the experiment was carried out in the presence of nigericin, which results in

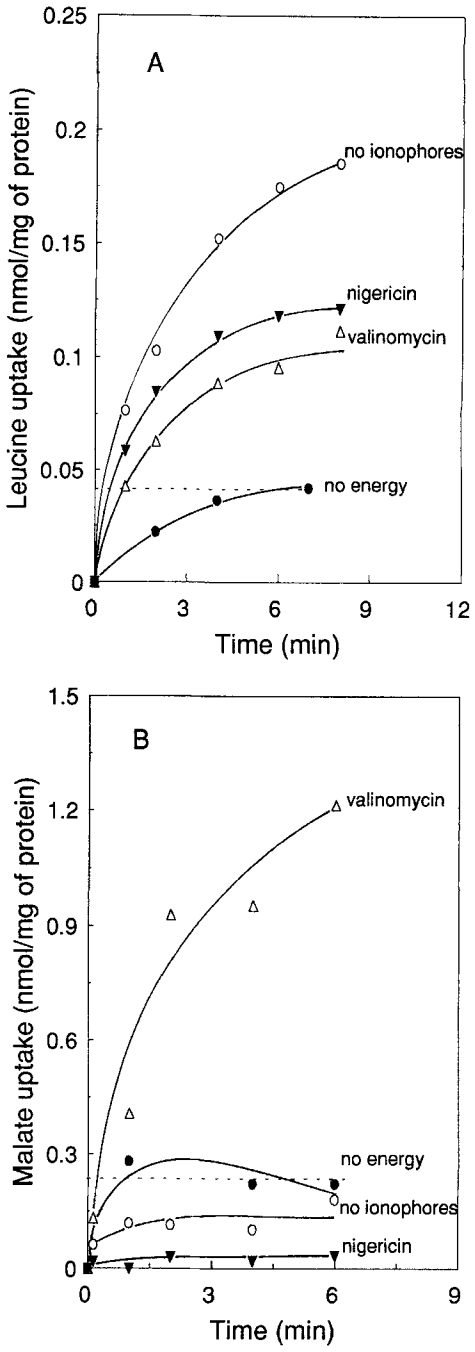

Fig. 1. Uptake of L-leucine (A) and L-malate (B) by hybrid membranes prepared from membrane vesicles of *L. oenos* 84.13 MLE(−) and proteoliposomes containing cytochrome *c* oxidase. L-[^{14}C]leucine (3.2 µM, final concentration) and L-[^{14}C]malate (20.8 µM, final concentration) uptake was assayed at 25°C in the presence of ascorbate-*N, N, N′, N′*-tetramethyl-*p*-phenylenediamine-cytochrome *c* in the absence (○) or presence (△) of 1 µM valinomycin or 0.5 µM nigericin (▼), at pH 5.0. Uptake of L-leucine or L-malate without ascorbate-*N, N, N′, N′*-tetramethyl-*p*-phenylenediamine-cytochrome *c* (●). Final protein concentrations were 0.36 mg/ml and 0.25 mg/ml for leucine and L-malate uptake, respectively.

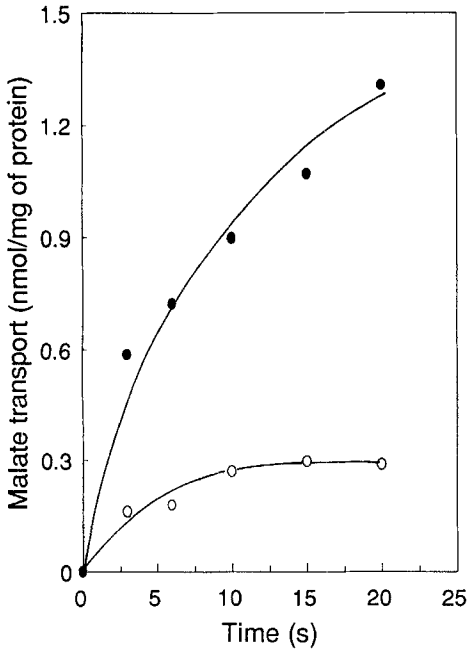

Fig. 2. Electrogenic uptake of L-malate in hybrid membranes prepared from membrane vesicles of *L. oenos* 84.13 MLE(−) fused with liposomes. Sodium loaded proteoliposomes (as described in Materials and Methods) were diluted into potassium phosphate (●) or sodium phosphate (○) supplemented with 1 mM benzoic acid and in the presence of 13.8 μM L-[^{14}C]malate and 1 μM valinomycin. Uptake was assayed at 25 °C. The final protein concentration was 0.07 mg/ml.

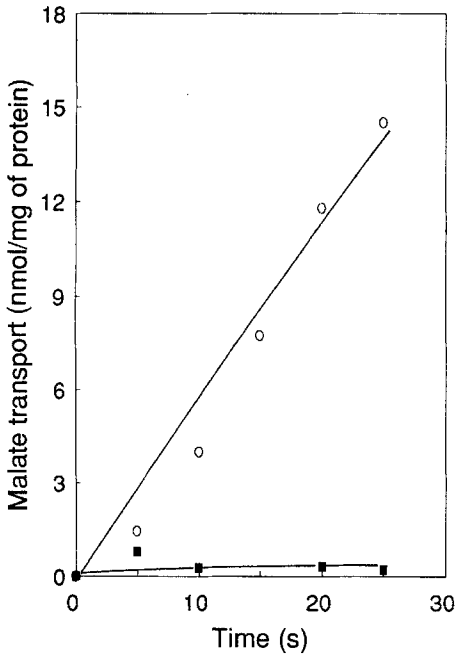

Fig. 3. Uptake of L-malate driven by a pH gradient in hybrid membranes prepared from *L. oenos* 84.13 MLE(−) membrane vesicles fused with liposomes. Sulfuric acid along with L-[^{14}C]malate (14.5 μM, final concentration) was added to the hybrid membranes suspended to a final protein concentration of 0.27 mg/ml in 50 mM potassium phosphate, pH 6.5. Uptake was assayed at 25 °C at a final pH of 3.4 (○). Nigericin (■) was added at a final concentration of 0.5 μM.

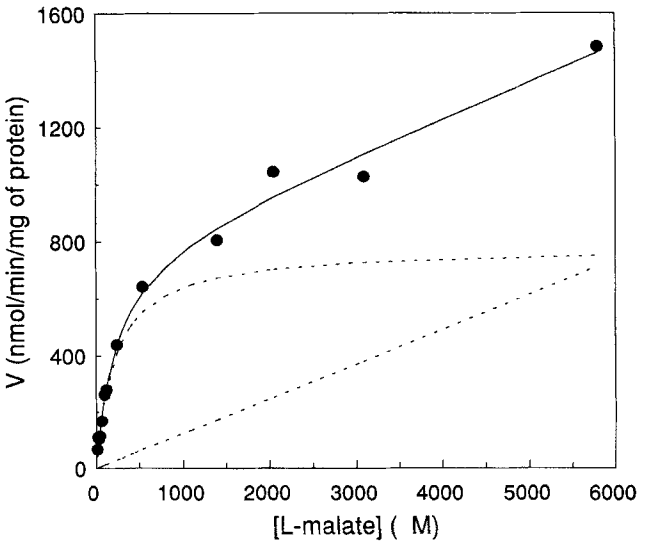

Fig. 4. Initial rates of L-malate uptake as a function of L-malate concentration. To generate an artifical pH gradient, sulphuric acid was added to fused membranes in 50 mM potassium phosphate, pH 6.5, (final protein concentration of 0.24 mg/ml) to yield a final pH of 4.0. The L-[^{14}C]malate concentrations varied from 12.5 μM to 5.8 mM. Uptake was assayed at 25 °C. The data were fitted to the equation: $V = V_{max}[S]/(K_m^{app} + [S]) + K_d[S]$ (solid line), in which V_{max} is the maximal rate of uptake, K_m^{app} is the apparent affinity constant, and K_d is the constant relating to the linear uptake component. The broken lines represent the Michaelis Menten and linear component of the L-malate uptake.

a rapid equilibration of protons thereby preventing the building up of a significant ΔpH and $\Delta\psi$, no accumulation of L-malate was observed. These results are consistent with the transport of a negatively charged substrate that can deprotonate (uniport of malateH$^-$), or the transport of dianionic malate in symport with one proton.

Kinetic analysis of malate transport

The kinetic parameters of L-malate uptake were determined in hybrid membranes in which an artificial pH gradient (inside alkaline) was generated. Initial rates of L-malate transport were estimated from the uptake of radioactivity over 2 s and in the concentration range 12.5 μM to 5.8 mM. The experimental data did not correspond with simple Michaelis-Menten kinetics but could be fitted with a saturable plus a linear term in a rate versus [L-malate] plot (Fig. 4). The kinetic parameters for malate transport, estimated from the saturable component, correspond with an apparent affinity constant (K_m^{app}) of 0.22 mM and a maximal rate V_{max} of 0.78 nmol · min^{-1} · mg^{-1} protein. The linear component, which only becomes manifest above an L-malate concentration of 1 mM, could represent passive diffusion or transport with a very low affinity ($K_m^{app} > 10$ mM). Estimation of the high-affinity component at different pH values in the range 3.0−5.8 yielded apparent affinity constants that varied more than 100-fold when calculated on the basis of the concentrations of either undissociated acid (L-malateH_2) or completely dissociated acid (L-malate^{2-}) (Table 1). The variation in K_m^{app} was less than twofold when expressed as the concentration of monoanionic L-malate (L-malateH^-), which is indicative for L-malateH^- as the transported species. It should be noted that the data are also consistent with a carrier protein

Table 1. Kinetic parameters of L-malate uptake in fused membranes of *L. oenos* as a function of pH. The experiments were performed as described in the legend to Fig. 4, except that the final pH was varied as indicated. The K_m^{app} values were calculated by substracting a linear component as indicated in Fig. 4; for the calculation of the K_m^{app} values on the basis of the individual chemical species pK_a values of 3.4 and 5.2 were used.

pH	V_{max}	K_m^{app} for			
		total malate	$malateH_2$	$malateH^-$	$malate^{2-}$
	μmol · min^{-1} · mg^{-1}	μM			
3.0	1.4	1029	735	292	2
4.0	0.8	219	42	167	11
5.1	1.1	542	6	298	237
5.8	0.4	898	1	180	717

that accepts all three species or one that accepts the monocarboxylate and dicarboxylate anions with the K_m^{app} going through a minimum at pH 4.0.

L-malate exchange and substrate specificity

Counterflow experiments were performed to establish whether the L-malate transport system could mediate homologous exchange of L-malate. L-malate-loaded hybrid membranes were concentrated and, subsequently, diluted 100-fold into the assay buffer containing L-[^{14}C]malate. Although an outwardly directed malate gradient was imposed, significant uptake of L-malate could not be detected. In another set of experiments, hybrid membranes, prepared from membrane vesicles that were fused with liposomes containing cytochrome *c* oxidase, were allowed to accumulate L-[^{14}C]malate in response to ΔpH and the release of L-malate upon addition of unlabelled substrate was monitored. High concentrations of L-malate (10 mM) were required for chasing internal L-[^{14}C]malate, corresponding to a low-affinity exchange reaction (Fig. 5). In comparison, the addition of nigericin caused a rapid release of L-[^{14}C]malate indicating that, under these conditions, the efflux reaction is more rapid than exchange in *L. oenos*. In the presence of nigericin, the internal pH becomes equal to the external pH, and, as a result, the fraction of monoanionic L-malate (transported species) increased, which may have caused the higher rates of efflux relative to exchange. To test this possibility, membrane vesicles of *L. oenos* were equilibrated with 5 mM L-malate and, subsequently, diluted into media without and with an equal concentration of L-malate and equal internal and external pH values. The rates of efflux and exchange were similar under these conditions (data not shown). In contrast, membrane vesicles of *L. lactis* loaded with L-[^{14}C]malate only showed a very rapid exit of L-malate in the presence of L-malate or L-lactate (data not shown). These exchange reactions were much more rapid than the efflux of L-[^{14}C]malate [15].

To determine whether malate uptake and lactate efflux could be coupled in an exchange reaction, lactate and some other carboxylic acids were tested for their ability to chase internal L-[^{14}C]malate (Fig. 6). Pyruvate and tartarate did not elicit exit of L-[^{14}C]malate nor did these compounds have an effect on L-malate uptake. D-lactate and L-lactate, in contrast

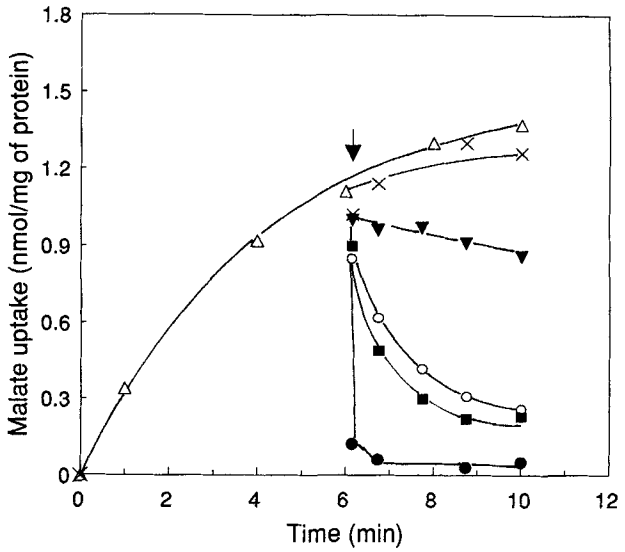

Fig. 5. Release of L-malate from hybrid membranes, prepared from membrane vesicles of *L. oenos* 84.13 MLE(−) fused with liposomes, upon addition of excess unlabelled L-malate. Hybrid membranes were allowed to accumulate L-[^{14}C]malate (7.3 μM, final concentration) for 6 min in the presence of the electron donor system (ascorbate-*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine-cytochrome *c*) plus 1 μM valinomycin and at pH 5.0 (△). The arrow indicates the addition of 1 (▼), 10 (○), 20 mM (■) L-malate, 20 mM Mes (potassium salt) (×) or 0.5 μM nigericin (●).

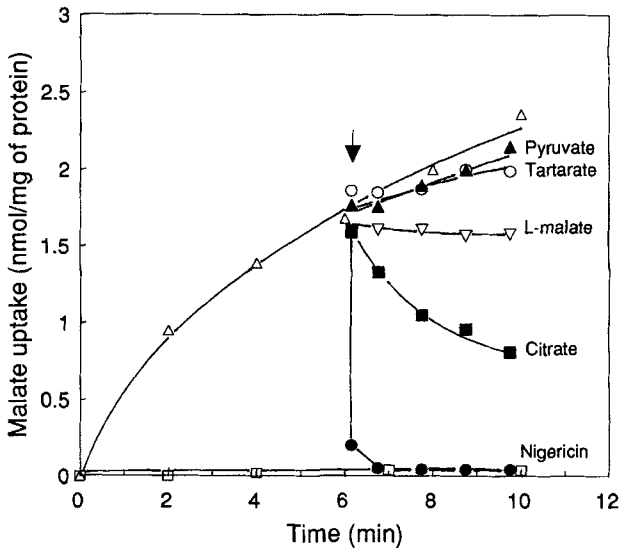

Fig. 6. Effect of unlabelled carboxylic acids on the release of L-malate from hybrid membranes prepared from membrane vesicles of *L. oenos* 84.13 MLE(−) fused with liposomes. Conditions were the same as specified in the legend to Fig. 4. The arrow indicates the addition of an 70-fold excess (0.5 mM) of unlabelled L-malate (▽), L-tartarate (○), pyruvate (▲), citrate (■) or 0.5 μM nigericin (●). Addition of L-lactate, D-lactate and acetate yielded the same effect as L-malate (represented by ▽). Nigericin added at $t = 0$ (□).

inhibited further uptake (accumulation) of L-[^{14}C]malate as did the same concentration of unlabelled L-malate. D-lactate and L-lactate at concentrations higher than 0.5 mM could not be tested for their ability to chase L-malate via an exchange mechanism, since diffusion of the weak acids caused dissipa-

tion of the ΔpH. Citrate elicited an efflux of L-malate that was even more pronounced than the efflux caused by the same concentration of L-malate, suggesting that a general carboxylate transport protein is operative in *L. oenos* with specificity for dicarboxylates, tricarboxylates and monocarboxylates.

DISCUSSION

In the present paper, we report the characterisation of malate transport in *L. oenos* MLE(−) using membrane vesicles fused with cytochrome-*c*-oxidase-containing liposomes. Transport of leucine and L-malate in response to the proton motive force (Δp) or its components ($\Delta\psi$ and ΔpH) was compared. For leucine uptake, the results are consistent with a leucine-H^+ symport mechanism as observed for *L. lactis* [5] and *Leuconostoc mesenteroides* [18]. L-malate uptake, in contrast, was found to be driven by a ΔpH (inside alkaline) and a $\Delta\psi$ (inside positive), indicating that the overall transport reaction involves the movement of a negative charge. This could mean that dianionic L-malate (L-malate^{2-}) is taken up in symport with one proton or that monoanionic (L-malateH^-) is transported via a uniport mechanism. The kinetic analysis of L-malate uptake at different pH values suggests a mechanism in which the monoanionic L-malate is transported. The driving force for L-malateH^- uptake by uniport is formed by the L-malateH^- concentration gradient. Since L-malateH^-dissociates into L-malate $^{2-}$ plus 1 H^+ in the vesicle lumen, the ΔpH acts as an apparent driving force on the transport system (as if it were a L-malate^{2-}-H^+ symport system). In whole cells, the driving force for the uptake of L-malate would be the chemical gradient of L-malateH^- that remains directed inwards by the pH gradient and the decarboxylation of L-malate inside the cell.

The analysis of the substrate specificity of the putative carboxylate carrier protein indicated that L-malate, D-lactate and citrate are substrates of the transport system. Kinetic analysis of the pH dependence of L-malate uptake (this study) and citrate uptake [16] suggests that both species are taken up in the monoanionic form. The malate and citrate transport activities in membrane vesicles of *L. oenos* are related irrespective a whether the cells are grown in medium supplemented with L-malate or citrate (M. Salema, unpublished results). This finding contrasts the situation in *L. lactis* where separate transport proteins are present for L-malate (Mal*P*) and citrate (Cit*P*) ([15]; unpublished results). In *L. lactis*, the *citP* gene is plasmid encoded whereas the *malP* gene is present on the chromosome (strain IL1403).

Whether possible exchange of L-malate for lactic acid plays a role during malolactic fermentation in *L. oenos*, as significant as that in *L. lactis*, is doubtful. The catalytic efficiency of the exchange reaction is not higher than that of the L-malate uniport reaction. L-malate transport in *L. lactis* by means of L-malateH^-/L-lactic acid exchange is at least two orders of magnitude greater than L-malate uniport, and the exchange reaction has been shown to occur under (physiological) conditions of L-malate fermentation in *L. lactis* [15].

Malolactic fermentation in *L. oenos* is associated with the generation of a Δp (Salema, M., Lolkema, J. S., San Romão, M. V. and Loureiro Dias, M. C., unpublished results). Most likely, the entry of monoanionic L-malate is followed by its decarboxylation in a reaction that consumes one proton to yield L-lactic acid plus carbon dioxide (Fig. 7). If one assumes that CO_2 and lactic acid leave the cell by simple electroneutral diffusion, the energy equivalent of one H^+ pumped out from the cytoplasm to the medium will be generated/molecule L-malate transported and decarboxylated. In a previous study, we have shown that metabolic energy is conserved from the metabolism of citrate in *L. oenos* in a manner similar to that of malolactic fermentation [16] (Salema, M., Lolkema, J. S., San Romão, M. V. and Loureiro Dias, M. C., unpublished results). This contribution suggests that the underlying mechanisms of metabolic energy conservation, i.e. electrogenic transport followed by intracellular proton consuming enzymic step(s) (Fig. 7), use the same transport protein to effect the uptake of monovalent carboxylates (L-malate or citrate) via electrogenic uniport.

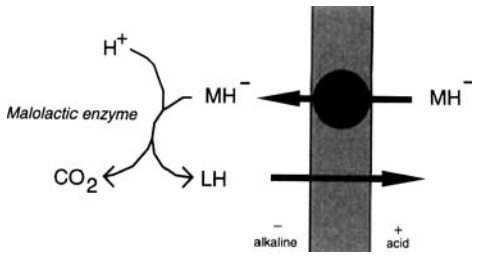

Fig. 7. Schematic representation of metabolic energy conservation by uniport of monoanionic L-malate (MH-) and L-malate decarboxylation.

L. oenos is able to maintain a rather constant intracellular pH (5.8−6.3) in the pH range 3.0−5.5 (Salema, M., Lolkema, J. S., San Romão, M. V. and Loureiro Dias, M. C., unpublished results). The highest L-malateH^- concentration gradient is achieved at pH 4.3, i.e. the pH at which this form predominates. Since the chemical gradient of L-lactic acid is low under conditions of malolactic fermentation at low pH (e.g. pH 3.5), there is, from a thermodynamic point of view, no advantage for the organism in catalysing an efficient L-malateH^-/L-lactic acid exchange reaction instead of L-malateH^- uniport in combination with passive L-lactic acid diffusion. In fact, at pH 3.5, the intracellular concentration of L-lactic acid inside is lower than that outside (pK_a of L-lactate is 3.8) (Salema, M., Lolkema, J. S., San Romão, M. V. and Loureiro Dias, M. C., unpublished results). Hence, we propose that uniport of L-malateH^- occurs in bacteria that ferment L-malate at relatively low pH, e.g. *L. oenos*, whereas in organisms such as *L. lactis* that carry out malolactic fermentation at higher pH values, the transport system has been optimized for L-malateH^-/L-lactic acid exchange.

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